

## Apoptosis after traumatic human spinal cord injury

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**Object.** Apoptosis is a form of programmed cell death seen in a variety of developmental and disease states, including traumatic injuries. The main objective of this study was to determine whether apoptosis is observed after human spinal cord injury (SCI). The spatial and temporal expression of apoptotic cells as well as the nature of the cells involved in programmed cell death were also investigated.

**Methods.** The authors examined the spinal cords of 15 patients who died between 3 hours and 2 months after a traumatic SCI. Apoptotic cells were found at the edges of the lesion epicenter and in the adjacent white matter, particularly in the ascending tracts, by using histological (cresyl violet, hematoxylin and eosin) and nuclear staining (Hoechst 33342). The presence of apoptotic cells was supported by staining with the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling technique and confirmed by immunostaining for the processed form of caspase-3 (CPP-32), a member of the interleukin-1 $\beta$ -converting enzyme/*Caenorhabditis elegans* D 3 (ICE/CED-3) family of proteases that plays an essential role in programmed cell death. Apoptosis in this series of human SCIs was a prominent pathological finding in 14 of the 15 spinal cords examined when compared with five uninjured control spinal cords. To determine the type of cells undergoing apoptosis, the authors immunostained specimens with a variety of antibodies, including glial fibrillary acidic protein, 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase), and CD45/68. Oligodendrocytes stained with CNPase and a number of apoptotic nuclei colocalized with positive staining for this antibody.

**Conclusions.** These results support the hypothesis that apoptosis occurs in human SCIs and is accompanied by the activation of caspase-3 of the cysteine protease family. This mechanism of cell death contributes to the secondary injury processes seen after human SCI and may have important clinical implications for the further development of protease inhibitors to prevent programmed cell death.

**KEY WORDS** • apoptosis • human spinal cord injury • caspase-3

**A**POPTOSIS is an important biological process in eukaryotes in which individual cells die by activating an intrinsic suicide mechanism. Apoptosis is distinguished from necrotic cell death by morphological and biochemical criteria. Apoptosis is an active process of cell destruction characterized by cell shrinkage, chromatin aggregation with genomic fragmentation, and nuclear pyknosis.<sup>27,31</sup> In contrast, necrosis is characterized by passive cell swelling, intense mitochondrial damage with rapid energy loss, and disruption of internal homeostasis. Necrosis leads to membrane lysis and release of intracellular constituents that evoke an inflammatory reaction.<sup>10,37,43</sup>

Apoptosis has long been known to occur as a form of neuronal cell death during embryonic development<sup>22,23</sup> and has been observed more recently following damage to the nervous system caused by ischemia, neurodegenera-

tive conditions, inflammatory diseases, and traumatic injuries.<sup>3,6,22,24,41,44,46,50,54</sup> Caspases are a family of cysteine proteases that play an important role in the effector phase of apoptosis. Caspase-3, in particular, has been shown to be important in neural development and injury. Germline deletions of this protease led to severe neurological defects in mice.<sup>34</sup> The contribution of caspase-3 activity and apoptosis to neuronal cell death after traumatic brain injury<sup>54</sup> and experimental transient ischemia<sup>24,41</sup> has been reported. In both injury paradigms, the use of caspase inhibitors not only reduced the extent of apoptosis, but also resulted in functional behavioral improvement in the animals.

The presence of apoptosis in spinal cord injury (SCI) following a contusion has been reported recently in rats and monkeys.<sup>11,32,35,36</sup> In these studies, it was shown that

TABLE 1  
Summary of the clinical data of the 15 patients with SCI\*

Case No.	Survival Time	Age (yrs), Sex	Injury Level	Cause of Injury	Neurological Status	Classification of Injury	Histopathological Findings at Lesion Epicenter	Presence & Location of Wallerian Degeneration
1	3 hrs	72, M	T-6	fall	complete	massive compression	maceration & hemorrhagic components	no wallerian degeneration
2	3 days	67, M	C-6	fall	incomplete	solid cord	minor increase in interstitial spaces	subtle, above
3	6 days	74, M	C6-7	MVA	complete	massive compression	all neural tissue damaged at epicenter	mild, above
4†	8 days	20, F	C7-8	GSW	complete	laceration	subtotal destruction of neural tissue at epicenter	moderate, above & below
5†	10 days	22, M	C-7	diving	incomplete	contusion	macerated tissue & phagocytic response at epicenter	mild, asymmetric; above & below
6	12 days	59, M	C-6	MVA	complete	contusion	major loss of neural tissue at epicenter	severe, above & below
7	12 days	16, F	T-11	GSW	complete	laceration	all neural tissue damaged at epicenter	very subtle, above & below
8	12 days	19, M	L-1	GSW	complete	contusion	macerated tissue at epicenter; vascular infarction proximal to epicenter	severe, up to C-3 & below
9	15 days	38, M	T-5	GSW	complete	laceration	spinal cord transected at T-5; necrotic tissue at T-4 w/ invasion of macrophages	moderate, above & below
10	16 days	43, M	C5-6	fall	complete	contusion	large blood-filled cavities w/ macrophage invasion at epicenter; preserved axon/myelin units in extreme periphery of most of cord	mild
11	16 days	80, M	C-6	fall	complete	contusion	large destruction of cord tissue w/ macrophages & hemorrhage	mild, more present above than below
12	17 days	70, M	C-8	MVA	complete	contusion	loss of neural tissue at epicenter	moderate, above & below
13	17 days	17, F	C6-7	MVA	complete	contusion	macerated tissue in ventral & central portions of cord at epicenter	moderate, more present above than below
14	45 days	67, M	C5-7	fall	complete	contusion	large destruction of cord tissue at epicenter	moderate, above & below
15	60 days	68, F	T-5	fall	incomplete	contusion	loss of motor neurons at epicenter & diffuse axonal injury throughout dorsal columns & lateral & ventral corticospinal tracts	severe in dorsal columns

\* GSW = gunshot wound; MVA = motor vehicle accident.

† Received methylprednisolone according to the Second National Acute Spinal Cord Injury Study protocol.

apoptosis contributed to the tissue damage seen after SCI. Apoptotic cell death was observed in both neurons and oligodendrocytes<sup>11,36</sup> and was prominent in the white matter, in which wallerian degeneration was simultaneously observed. A time course analysis in rats<sup>36</sup> revealed that apoptosis occurred as early as 4 hours postinjury and could be seen in decreasing amounts as late as 3 weeks after SCI.

We initiated this study to determine whether apoptosis is an important factor after human SCI by examining the spinal cords of patients who died between 3 hours and 2 months postinjury. Apoptosis in these 15 patients was assessed using multiple criteria, including nuclear morphology, chromatin staining techniques, and immunostaining for a processed form of caspase-3. We also determined the spatial and temporal expression of apoptotic cells and the nature of the cells involved in programmed cell death.

### Clinical Material and Methods

#### Patient Population

This study was based on postmortem examination of spinal cord tissue from five control patients without SCI and 15 patients who died at different time points after an SCI. Fifteen specimens were obtained from the Miami Project's Human Spinal Cord Injury Bank, which contains more than 100 injured human spinal cords. The cases were

selected based on the short interval between SCI and death (0–2 months) and the availability of tissue above and below the lesion site.

#### Preparation of Specimens

The spinal cords had been removed at autopsy, usually within 16 hours after death. They were then fixed in 10% neutral buffered formalin for a minimum of 15 days, after which they were stored in 0.1 M phosphate buffer at 4°C. Selected tissue blocks were taken at the epicenter and at three consecutive root levels above and below the injury. Samples from each of these regions were dehydrated, embedded in paraffin, and cut in 7-μm-thick cross-sections.

Sections were stained with hematoxylin and eosin and cresyl violet (15 samples), terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate nick-end labeling ([TUNEL], five samples), or Hoechst 33342 (four samples), and immunostained with CM 1, an antibody that preferentially recognizes the processed form of caspase-3 (10 samples). Sections from control spinal cords (five samples) were stained with cresyl violet and immunostained with the CM 1 antibody. Wallerian degeneration in white matter tracts was assessed using a silver stain for axons (Sevier-Munger) and a myelin stain (solochrome-cyanine) on adjacent sections. Regions undergoing wallerian degeneration contained swollen, fragmented, or absent axons in the same areas in which myelin

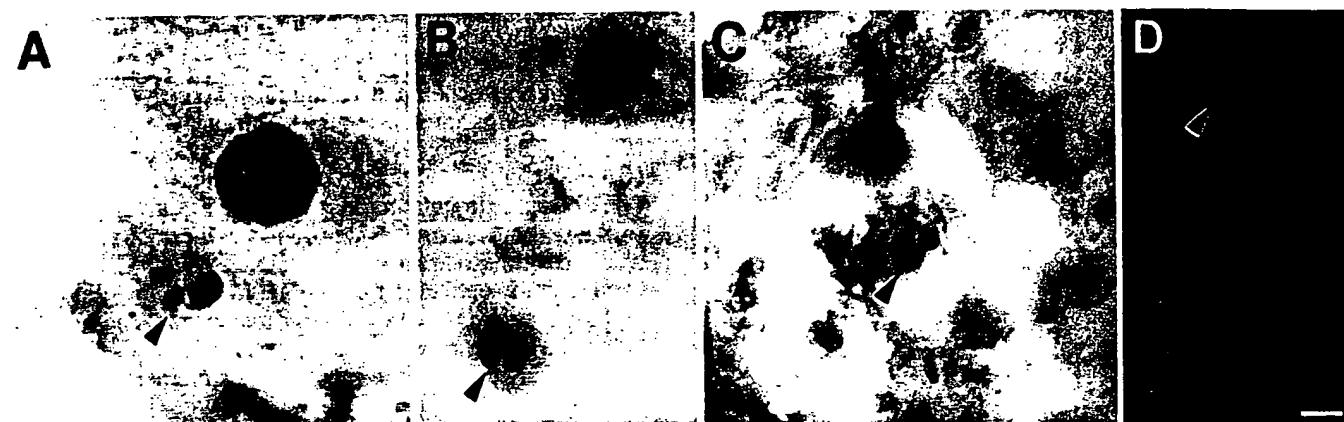


FIG. 1. Photomicrographs showing apoptotic nuclei (arrowheads) within the traumatized human spinal cord stained with cresyl violet (A and B), H & E (C), and Hoechst 33342 (D). Apoptotic nuclei appear as condensed, clumped fragments. Bar = 20  $\mu$ m.

was distorted or collapsed. Wallerian degeneration was quantified in the white matter above and below the lesion according to four levels of severity: subtle, mild, moderate, and severe.

#### *Staining With the TUNEL Technique*

To determine whether DNA fragmentation characteristic of apoptosis occurs, we stained spinal cord tissue by using the *in situ* TUNEL technique.<sup>19</sup> Spinal cord sections were deparaffinized in xylene for 5 minutes and then rehydrated sequentially in 100%, 95%, 75%, and 50% ethanol. The sections were incubated with 50  $\mu$ g/ml proteinase K for 5 minutes to strip off nuclear proteins. The TUNEL staining was completed using the apoptosis *in situ* kit according to the manufacturer's instructions.

Briefly, sections were immersed in equilibration buffer for 10 minutes and then incubated with TdT and deoxyuridinetriphosphate-fluorescein isothiocyanate in a humidified chamber at 37°C for 1 hour. The sections were

washed with 0.1 M phosphate-buffered saline (PBS) at pH 7.4 and counterstained with propidium iodide (a nuclear stain) for 10 minutes. As a positive control we pretreated slides with DNase to produce TUNEL-positive staining of all nuclei, and the negative controls were incubated without TdT enzyme. Sections were examined and photographed using fluorescence microscopy.

#### *Hoechst 33342 Staining*

Spinal cord sections were deparaffinized with xylene for 5 minutes, rehydrated, and rinsed with 0.1 M PBS. The sections were stained with one drop of glycerol/PBS solution containing 5  $\mu$ l Hoechst 33342 dye (5.6 mg in 10 ml PBS).

#### *Immunostaining for Processed Form of Caspase-3 With the CM 1 Antibody*

Spinal cord sections were deparaffinized as described earlier, rinsed in 0.01 M PBS, and treated for 30 minutes

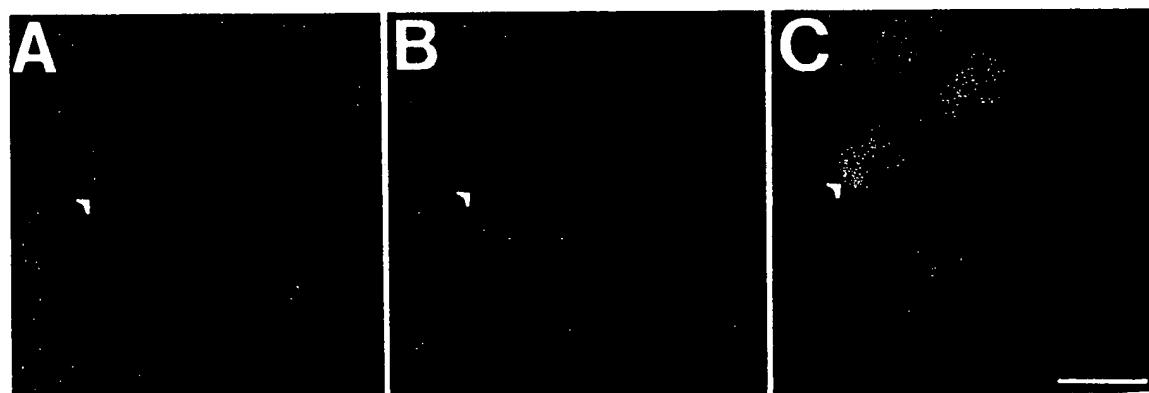


FIG. 2. Photomicrographs showing TUNEL-labeled cells in the spinal cord at the epicenter level. Cross-sections of the spinal cord demonstrate a single TUNEL-positive labeled nucleus that appears small and fragmented (A and B, arrowhead), among numerous normal nuclei counterstained by propidium iodide nuclear dye (B). On double exposure (C), the apoptotic cell is yellow (arrowhead), whereas the normal nuclei remain red. Bar = 50  $\mu$ m.

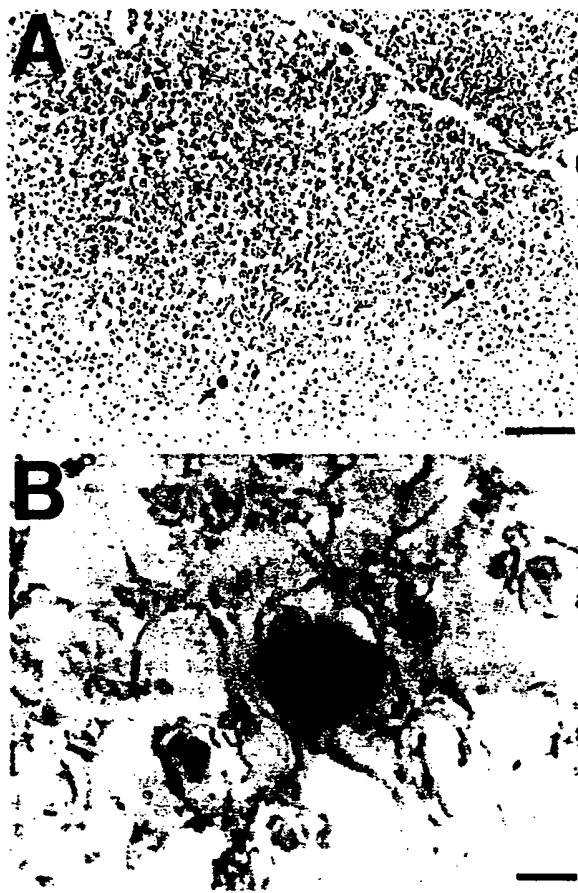


FIG. 3. Photomicrographs showing CM 1 immunostaining of the dorsal columns at low (A) and high magnification (B) demonstrating a number of dark and shrunken cells (arrows) containing condensed and clumped chromatin. The staining for processed CPP-32 is evident within the nucleus (B). Bar = 200  $\mu\text{m}$  (A), 10  $\mu\text{m}$  (B).

with 3%  $\text{H}_2\text{O}_2$  in 10% methanol. The sections were blocked for 1 hour in 5% normal goat serum with 0.1 M PBS and 0.1% Triton X-100. The primary antibody CM 1 (0.012  $\mu\text{g}/\text{ml}$ ) was applied and the preparations were left overnight. The following morning, the sections were fixed for 10 minutes in 0.01 M PBS containing 0.1% glutaraldehyde. The sections were incubated for 1 hour with a biotinylated secondary antibody, followed by a 1-hour incubation with an avidin-biotin complex (ABC) reagent. Sections were then incubated for 10 minutes in 0.5% biotinylated tyramide 1%  $\text{H}_2\text{O}_2$  solution in 0.01 M PBS. They were reincubated<sup>1</sup> in the ABC reagent for 30 minutes. The stain was developed in diaminobenzidine and  $\text{H}_2\text{O}_2$  and enhanced by nickel chloride in PBS. The sections were rinsed, dehydrated, mounted on a coverslip, and examined under the light microscope. Sections from uninjured control cases were processed using the same protocol.

#### Immunostaining for Glial Fibrillary Acidic Protein, CNPase, CD45, and CD68

Immunohistochemical techniques were used to detect the following antigens: glial fibrillary acidic protein (GFAP), 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNPase), and CD45/CD68. Glial fibrillary acidic protein GFAP is an astrocyte marker; CNPase is a myelin protein in the central nervous system and has been shown to be specific for oligodendrocytes;<sup>45</sup> and CD45 and CD68 were used to study activated microglia and macrophages.

Tissue sections were deparaffinized and endogenous peroxidase activity was blocked with 0.3%  $\text{H}_2\text{O}_2$  in 100% methanol. Sections were incubated with either 0.3% Triton X-100 in 0.1 M PBS (GFAP) or with Tris (pH 7.6) containing 10% normal goat serum. The sections were incubated overnight at room temperature with the following monoclonal antibodies: GFAP 1:200, CD45 1:100, CD68 1:100, and CNPase 1:800. This was followed by sequential incubation with biotinylated secondary antibody, ABC with horseradish peroxidase, 3,3'-diaminobenzidine, and  $\text{H}_2\text{O}_2$ . The slides were then counterstained with 2% cresyl violet, dehydrated, and mounted. Omission of the primary antibody served as a negative control.

#### Sources of Supplies and Equipment

The proteinase K and Hoechst 33342 dye were purchased from Sigma Chemical Co., St. Louis, MO. The apoptosis in situ kit was acquired from Chemicon International, Inc., Temecula, CA. The Axiophot fluorescent microscope was obtained from Zeiss, Inc., Oberkochen, Germany. The primary antibody CM 1 was a generous gift from IDUN Pharmaceuticals, Inc., La Jolla, CA. The biotinylated secondary antibody and ABC reagent (Vectastain) were acquired from Vector Laboratories, Burlingame, CA. The monoclonal antibodies GFAP, CD45, and CD68 were purchased from Dako Corp., Carpenteria, CA, and the CNPase from Sternberger Monoclonals, Inc., Baltimore, MD.

## Results

#### Clinical Data

The mean age of the 15 patients with SCIs (Table 1) was 49 years (range 16–80 years) and the mean age of the five control patients was 55 years (range 33–77 years). The principal causes of death in the patients with SCIs were pulmonary embolism (six patients), pneumonia (six patients), hemothorax with cardiac arrest in one, and an unknown cause in two patients. The causes of death for the control patients were cardiovascular disease in two and a motor vehicle accident (without head injury or SCI) in three. Only two patients with SCI (Cases 4 and 5) received methylprednisolone according to the Second National Acute Spinal Cord Injury Study protocol.<sup>8</sup>

Ten patients suffered a cervical and four a thoracic SCI, and one case consisted of a conus medullaris injury. Twelve patients presented with a complete motor and sensory loss below the level of their injury, whereas three experienced incomplete loss. The cause of injury was variable and included falls (six patients), motor vehicle

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TABLE 2  
Distribution and quantification of apoptotic cells per section<sup>†</sup>

Case No.	Injury Level	Apoptotic Cells	Epicenter Level	1-3 Levels Above Epicenter	1-3 Levels Below Epicenter
1	T-6	yes	no viable tissue	normal tissue* T-5; no apoptotic cells above T-5	normal tissue; no apoptotic cells
2	C-6	yes	edges of lesion*	dorsal columns*	ventral CST, lat CST*; no apoptotic cells below C-8
3	C6-7	yes	no viable tissue	dorsal columns**	at edges of central cavity*
4	C7-8	yes	edges of lesion**	dorsal columns (fx gracilis)*	ventral CST (T-1)*; no apoptotic cells below T-2
5	C-7	yes	edges of cavity*	no apoptotic cells from C5-7	dispersed at C-8*; no apoptotic cells at T-1 & below
6	C-6	yes	edges of lesion*	dorsal columns, spinocerebellar tracts**	ventral CST*
7	T-11	yes	edges of lesion*	dorsal columns*	dispersed T12-L1*; no apoptotic cells below L-2
8	L-1	yes	no viable tissue	dorsal columns (T-11) & a few cells in CST & spinocerebellar tracts***	ventral CST L3-5**
9	T-5	yes	no viable tissue	spinothalamic tract (T-3)*	no apoptotic cells T4-8
10	C5-6	no	—	—	—
11	C-6	yes	edges of lesion**	dorsal columns (fx gracilis); spinothalamic tracts*	ventral CST**
12	C-8	yes	edges of lesion**	dorsal columns & spinocerebellar tracts**	no apoptotic cells at T-1 & below
13	C6-7	yes	edges of lesion***	dorsal columns & spinothalamic tracts**	ventral CST & lat CST**
14	C5-7	yes	edges of lesion*	dorsal columns*	ventral CST & lat CST*
15	T-5	yes	edges of lesion*	dorsal columns*; no apoptotic cells above T-3	ventral CST*

<sup>†</sup> Asterisks represent the stratification of cell counts per section: \* = 1-15; \*\* = 15-30; \*\*\* = 30-45. Abbreviations: CST = corticospinal tract; fx = funiculus; — = not applicable.

accidents (four patients), gunshot wounds (four patients), and a diving accident (one patient).

### Histological Data

We analyzed cross-sections of the lesion epicenter and rostral and caudal levels in tissue obtained in 15 patients who died between 3 hours and 60 days after their SCI. According to the classification established by Bunge, et al.,<sup>9</sup> nine patients presented with a contusion/cavity lesion, two with a massive compression, three with a laceration lesion, and one with a solid spinal cord syndrome.

All patients except the one in Case 2 showed significant destruction of the spinal cord parenchyma at the epicenter of the injury. Signs of wallerian degeneration in the white matter occurred as early as Day 3 postinjury and thereafter spread rostral and caudal to the lesion, with more advanced degeneration of the ascending tracts. In one patient (Case 8), the contusion was associated with a vascular infarction proximal to the epicenter and a pattern of obvious wallerian degeneration.

### Morphological Appearance of Apoptotic Cells

Apoptotic nuclei were found in 14 of the 15 cases on hematoxylin and eosin- and cresyl violet-stained sections (Fig. 1A-C). Cells containing apoptotic nuclei exhibited evidence of cytoplasmic shrinkage. The nuclei themselves demonstrated chromatin condensation or aggregation into small and dark nuclear fragments. Staining with Hoechst 33342 revealed typically small and bright fragmented nuclei (Fig. 1D). No apoptotic bodies were detected on cresyl violet-stained sections from the five uninjured control patients.

The *in situ* TUNEL technique (Fig. 2A) combined with propidium iodide (Fig. 2B) also demonstrated apoptotic

TUNEL-positive nuclei as double-stained (yellow), fragmented nuclei (Fig. 2C) among numerous propidium iodide-positive cells. The location and quantity of apoptotic nuclei observed using this technique correlated with the number of apoptotic nuclei observed with hematoxylin and eosin- and cresyl violet-stained sections (paired t-test,  $p = 0.16$ ; correlation coefficient 0.83).

The presence of apoptotic cells was also demonstrated in sections immunostained with the CM 1 antibody. Most positive nuclei were dark and shrunken and contained condensed, clumped chromatin (Fig. 3A and B). Relatively more apoptotic cells were detected after CM 1 antibody staining compared with cresyl violet staining. As cresyl violet staining was used to detect apoptotic bodies and caspase-3-positive cells did not necessarily contain these bodies, the implication is that immunostaining for caspase-3 activation detects apoptosis at an earlier stage. Staining with caspase-3 was also negative in Case 10, in which no apoptotic cells were shown in cresyl violet-stained sections. Staining with caspase-3 showed one to two positive cells per section in tissue obtained in one control patient (73 years old, died of cardiovascular disease), whereas tissue from the four other uninjured control patients was negative. Other potential explanations for the few apoptotic cells seen in tissue obtained in the one control case are the patient's advanced age and associated vascular disease.

### Temporal and Spatial Distribution of Apoptotic Cells

Apoptotic cells were seen between 3 hours and 2 months postinjury. Although too few cases were analyzed to allow a correlation between the time after SCI and the number of apoptotic cells, most of these cells were seen in tissue obtained in patients who died between 3 days and 3

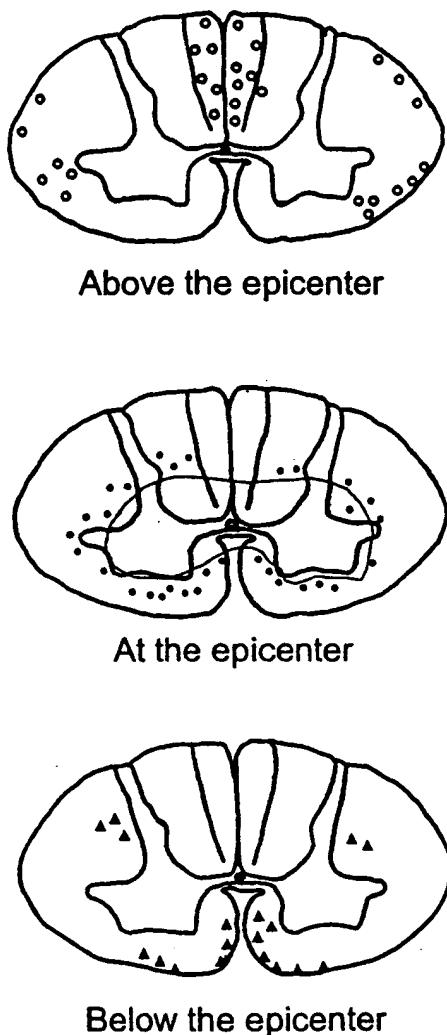


FIG. 4. Schematic diagram demonstrating the spatial distribution of apoptotic cells in the spinal cord of the patient in Case 13, who died 17 days postinjury. The apoptotic bodies were identified on cresyl violet-stained sections and are represented as either filled circles at the epicenter level, empty circles above, or filled triangles below the lesion.

weeks after the SCI. Incomplete neurological injury was associated with fewer apoptotic bodies (Cases 2, 5, and 15). When ischemia was associated with the primary injury, as in Case 8, a large number of apoptotic nuclei were observed throughout the levels studied.

Most apoptotic cells were randomly distributed in the rim of surviving tissue around the epicenter of the SCI and within the adjacent white matter (Table 2), and none was identified in the gray matter. Apoptotic cells were seen in areas of wallerian degeneration in the white matter above and below the epicenter. Rostral to the lesion epicenter, apoptosis was associated with axonal degeneration in ascending tracts, especially the funiculus gracilis, the spinoreticular, the spinothalamic, and the spinocerebellar tracts. Degeneration and apoptosis were absent in the regions containing descending motor pathways. Apoptosis was associated with the degenerating axons in the de-

scending tracts caudal to the lesion, especially the ventral corticospinal, reticulospinal, and vestibulospinal tracts; it was less prevalent in the lateral corticospinal or rubrospinal, and no apoptotic cells were seen in the caudal ascending tracts. Apoptotic cells were seen in much larger numbers in ascending than in descending tracts, and wallerian degeneration was present earlier in the ascending tracts than in the descending tracts. There appeared to be a good correlation between apoptosis and wallerian degeneration. A schematic diagram (Fig. 4) demonstrates the spatial distribution of apoptotic cells at the SCI epicenter as well as above and below the lesion in the spinal cord of the patient in Case 13, who died 17 days postinjury.

#### Apoptotic Cell Type

To identify the type of cells undergoing apoptosis, different antibodies directed against astrocytes (GFAP), oligodendrocytes (CNPase), and macrophages or activated microglia markers (CD45, CD68) were assayed. Apoptotic bodies were not seen in astrocytes (data not shown). Immunostaining with CNPase demonstrated that apoptotic cells (identified using cresyl violet counterstain) were present within oligodendrocytes adjacent to myelin sheaths in degenerating white matter tracts (Fig. 5). Macrophages or activated microglia were seen engulfing fragments of apoptotic cells. Apoptotic bodies in proximity to cells unstained with microglia markers were also observed (Fig. 6).

#### Discussion

Apoptosis is a form of physiological cell death, also defined as programmed cell death, in which cells die and are engulfed by phagocytes without discharging cytosolic contents into the extracellular space and without initiating an inflammatory reaction.<sup>10</sup> The cell surface membrane begins to form blebs and express pro-phagocytic signals, the cell shrinks and severs contact with its neighbors, chromatin becomes condensed and cleared, and eventually the whole cell fragments into membrane-bound vesicles that are rapidly ingested by neighboring cells. The apoptotic process can be rapid when compared with necrotic cell death, and the debris is removed with similar swiftness.<sup>17,37</sup> Because the process is rapid, quantification of the number of apoptotic cells on any given cross-section underestimates the extent to which apoptosis contributes to the death of cells at the injury site.<sup>52</sup>

The genetic control and the biochemical markers of apoptotic cell death were initially elucidated in the roundworm *Caenorhabditis elegans*, and *CED 3* was identified as a gene encoding a protein involved in programmed cell death in this maturing roundworm.<sup>29</sup> The mammalian homologs of cell death mechanisms consist of the *CED 3/ICE* (interleukin-1 $\beta$ -converting enzyme) family of cysteine proteases (caspases),<sup>17,38,40</sup> in which the prototype is the ICE. The mammalian caspase family is composed of at least 10 known members.<sup>2</sup> One of these is caspase-3 (Yama, apopain), which has been definitively implicated in apoptosis.<sup>16,26</sup> The essential role of caspases in vertebrate apoptosis is consistent with their activity as the principal effectors of apoptosis through their proteolytic

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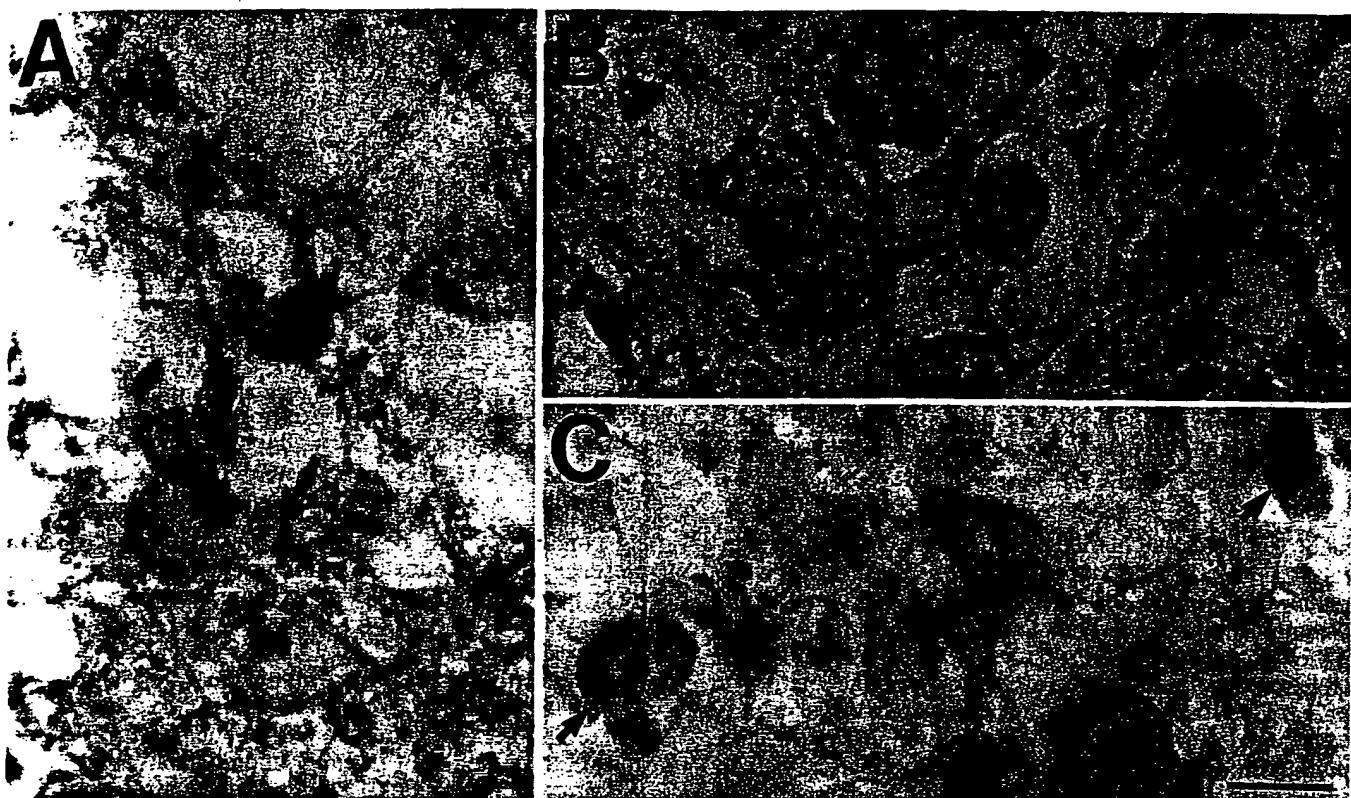


FIG. 5. Photomicrographs showing oligodendrocytes immunostained with CNPase and nuclei counterstained with cresyl violet. Normal oligodendrocytes are seen (A), as well as multiple apoptotic oligodendrocytes (arrows) (B and C). Bar = 50  $\mu$ m.

action on specific targets. One of the final effectors of cell death is activation of endonucleases that induce fragmentation of nuclear DNA into 185-bp fragments.<sup>30</sup> In a number of reports caspases have been implicated as important during apoptosis of neurons and astrocytes, and it has been suggested that caspase-3 was the principal effector in the apoptotic pathway.<sup>4,33</sup> Moreover, caspase-3 activation was never observed in necrotic cell death.<sup>4,26</sup> Hisahara, et al.,<sup>26</sup> demonstrated that caspase-1 (ICE) and caspase-3 (CPP-32) were expressed in oligodendrocytes and that their inhibition prevented apoptotic cell death.

Secondary injury processes are believed to be an important, remediable component of SCI.<sup>8,49</sup> Many of the basic research advances that have reached the clinical arena focus on the prevention of these secondary injury mechanisms.<sup>21,55</sup> Apoptotic cell death has been recognized for many years; however, this process has now gained increasing attention in the basic science literature as a mechanism by which cells die in a number of neurological diseases.<sup>47</sup> Apoptotic cell death appears to be yet another mechanism in which cells may die in a delayed fashion after injury, that is, a secondary injury mechanism.

There is evidence that apoptotic cell death contributes to tissue damage, and prevention of this process results in neurological recovery after SCI<sup>36</sup> and brain injury in rats.<sup>54</sup> Data presented here indicate for the first time that apoptosis is associated with the tissue damage observed after human SCI. Our determination of apoptosis relied on mul-

tiple criteria: morphological staining (cresyl violet, hematoxylin and eosin), nuclear chromatin staining with Hoechst 33342 dye, and the TUNEL test, all of which have been widely used for assessment of apoptosis. These results were confirmed using immunostaining with the CM 1 antibody that is specific for the processed form of caspase-3 (A Srinivasan, et al., unpublished data). Caspase-3 is required for DNA fragmentation and the morphological changes associated with apoptosis.<sup>30</sup>

Crowe, et al.,<sup>12</sup> presented the first evidence for the presence of apoptosis in SCIs in the rat. Li, et al.,<sup>35</sup> demonstrated that compression trauma to the spinal cord was associated with apoptosis of glial cells preferentially located in degenerating longitudinal tracts of the white matter. The apoptotic cells were most likely oligodendrocytes, a conclusion based on morphological data and negative GFAP staining. Further studies demonstrated the occurrence of apoptosis in SCI in rats and monkeys and showed that oligodendrocytes were the major cell population undergoing apoptosis based on immunohistochemical analysis.<sup>11,35</sup> Apoptosis of oligodendrocytes was seen in areas of Wallerian degeneration and was detected from 24 hours to 3 weeks postinjury.<sup>11,12</sup> Liu, et al.,<sup>36</sup> also observed a burst of neuronal and glial apoptosis in gray and white matter at the lesion site within the first 24 hours postinjury and a delayed wave of oligodendrocyte apoptosis in distant white matter several days later.

In our study, the lesion epicenter demonstrated the pres-



FIG. 6. Photomicrographs showing activated microglia immunostained with CD68 before the section was counterstained with cresyl violet to demonstrate apoptotic bodies. Cells that did not stain with the activated microglia marker (A, arrow) as well as CD68-positive cells ingesting apoptotic bodies (B, arrow) were observed. Bar = 50  $\mu$ m.

ence of multifocal hemorrhages and necrotic tissue involving the central gray matter and the contiguous white matter. Apoptotic cells were identified surrounding the lesion epicenter. As early Wallerian degeneration occurred in the white matter tracts, a second phase of apoptotic cells appeared and these were positive for an oligodendrocyte marker (CNPase). Oligodendrocyte apoptosis was clearly associated with Wallerian degeneration and was more obvious in ascending than in descending tracts. This corresponds to the pattern of progression of Wallerian degeneration in which the ascending tracts show signs of degeneration before the descending tracts.<sup>9</sup>

We found no relationship between the average number of apoptotic bodies and time from injury because the injury mechanism and severity were different in each case. Nonetheless, it seemed that apoptosis was less severe in patients with incomplete neurological injuries. Liu, et al.,<sup>36</sup> demonstrated that after rat SCI, apoptotic glial cells were more abundant above than below the site of compression and that apoptotic cells were more numerous after moderate and severe injury compared with mild compression. We found no evidence of apoptosis within the spinal cord neurons. This indicates that any neuronal loss was the result of necrosis rather than apoptosis or that it occurred at an early stage before we could detect it. Tissue in only one of our cases was examined within 24 hours of death. Li, et al.,<sup>35</sup> demonstrated that neuronal apoptosis was complete within the first 24 hours, whereas Liu and colleagues<sup>36</sup> saw no evidence of apoptosis in the spinal cord neurons at 4 hours or 1, 4, and 9 days after compression trauma. We suspect that activated microglia (macrophages) clear apoptotic bodies, as has been observed by others,<sup>13,14,48,51</sup> but may also be partly responsible for the induction of apoptosis by secreting cytotoxic substances such as cytokines (tumor necrosis factor- $\alpha$ ) and nitric oxide.<sup>53</sup> Other investigators have also demonstrated that microglia can undergo apoptosis following damage to the nervous system.<sup>20,42</sup>

The mechanisms responsible for oligodendrocyte apoptosis remain unclear. It may occur as a result of loss of axonally derived survival signals (Wallerian degeneration)<sup>9</sup> and/or as a result of evolving adverse changes in the cellular milieu<sup>13</sup> resulting in axonal demyelination. If these axons are in continuity across the injury site, electri-

cal conduction will be impaired through the axon. The presence of demyelinated axons around the epicenter of SCIs has received attention recently.<sup>7,8</sup> One therapeutic strategy for SCIs relies on the use of the drug 4-amino pyridine (a potassium channel blocker) to enhance axonal conduction through areas of demyelination.<sup>23</sup> Thus, prevention of axonal demyelination after SCI by the reduction of apoptotic oligodendrocyte cell death may result in an overall reduction of partially injured axons.

The contribution of CPP-32 activity and apoptosis to neuronal cell death after traumatic brain injury<sup>54</sup> and experimental transient ischemia<sup>24,41</sup> has been reported. In both injury paradigms, the use of caspase inhibitors not only reduced the extent of apoptosis, but also resulted in functional behavioral improvement in the animals. In that regard, therapeutic interventions aimed at blocking apoptosis may be useful in reducing tissue damage after SCI and ultimately in improving functional outcomes. Liu, et al.,<sup>36</sup> reported that intraperitoneal injections of cycloheximide, a protein synthesis inhibitor, improved behavioral outcomes after spinal cord contusion injury in rats. Partial inhibition of protein synthesis can induce the production of *Bcl-2*,<sup>28</sup> an antiapoptotic human protooncogene important in cell survival that was shown to be upregulated in injured axons of the white matter following compression injury of the spinal cord.<sup>18,35</sup> Hisahara, et al.,<sup>26</sup> demonstrated that caspases were involved in tumor necrosis factor-mediated cell death of oligodendrocytes and that inhibition of these proteases can prevent apoptosis. Milligan, et al.,<sup>39</sup> identified peptide inhibitors of the ICE protease family that arrest programmed cell death of motor neurons in vivo and in vitro. In that regard, it would be useful to test drugs that inhibit caspases in the treatment of SCI.

### Conclusions

This work demonstrates for the first time that apoptotic cell death is observed from 3 hours to 8 weeks after traumatic human SCIs. Apoptosis occurs around the lesion epicenter as well as within areas of Wallerian degeneration in both ascending and descending white matter tracts. Oligodendrocytes were definitely implicated as cells undergoing apoptosis on sections of injured spinal cord in which immunohistochemical markers were used. Apop-

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tosis after human SCI appears to be dependent on activation of CPP-32. Inhibition of this process may have potential therapeutic benefits for reducing tissue damage and improving the outcome after SCI.

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